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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Wnt proteins are a family of 19 secreted glycoproteins that regulate numerous aspects of mammalian development. The functions of one member of this family, Wnt4, include roles in ductal branching of the mammary gland. Its role is the breast is of significant importance because it mediates hormone signals during pregnancy and its overexpression leads to hyperplasia and premature alveolar differentiation. Previous studies have identified key components of the Wnt signaling pathway including Frizzled proteins and LDL receptor-related proteins (LRPs) as receptors for this pathway. Although these receptors have been identified, the specificity of Frizzleds and LRPs for Wnt proteins is unclear and receptors involved in Wnt4 signaling in the breast are not known. Identifying the Wnt4 receptor will be crucial to understanding the mechanism by which overexpression of Wnts leads to hyperplasia and tumorigenesis. We have currently analyzed Frizzled and LRP expression at different stages of mammary development and in cell lines that respond/do not respond to Wnt4. We transiently transfected all receptors into Wnt4 non-responder cells and found two candidate Wnt4 receptors. These candidates were stably expressed in non-responder cells and shown to activate signaling in the presence of Wnt4.

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Table of Contents

Introduction

Wnt proteins comprise a family of 19 secreted glycoproteins that participate in a number of crucial developmental processes including cell proliferation, cell fate determination and differentiation of tissues such as the mammary gland (Cadigan and Nusse, 1997). Many Wnts signal through a well characterized pathway that begins when Wnts bind to cell surface receptors of the Frizzled and LDL receptor related protein (LRP) famlies and results in the stabilization of cytosolic β-catenin, formation of β-catenin/TCF complexes in the nucleus and regulated transcription of target genes. Constitutive activation of this pathway is one of the most common signaling abnormalities in human cancers (Polakis, 2000). Overexpression of some Wnt proteins has been reported in human breast tumors (Bergstein and Brown, 1999) and their overexpression in the mouse mammary gland leads to hyperplasia and subsequent tumors (Nusse and Varmus, 1992). Several Wnts, including Wnt4, are expressed during normal mammary development. Wnt4 is of particular interest because it mediates hormonal signals required for mammary differentiation during pregnancy and its expression is induced by progesterone during pregnancy (Brisken, 2000). Absence of Wnt4 results in reduced or delayed ductal branching. In contrast, overexpression leads to hyperplasia with increased ductal branching and premature alveolar differentiation similar to that seen with inappropriate expression of Wnt1 in the mouse mammary gland (Edwards, 1992, Bradbury, 1995, Robinson, 2000). Understanding the specific functions of Wnt4 at the cellular level would be greatly enhanced by identifying the specific Wnt4 receptor in the breast and this knowledge is crucial for understanding its role in hyperplasia and tumorigenesis. There is considerable evidence that Frizzled proteins and LRPs are Wnt receptors but there is little evidence for the specificity of these receptors for individual Wnt proteins. Signal transduction through the canonical pathway causes increases in B-catenin which can be assayed by Western blotting. Using this assay, we identified cell lines that respond differentially to Wnt4 and proposed that unresponsive cells lack specific Wnt4 receptors. The experiments in this research project seek to use this knowledge to identify the principal Wnt4 receptor and will exploit this knowledge to antagonize Wnt4 signaling in the breast.

Body

The purpose of the research is the identify the proteins on the cell surface that act as specific Wnt4 receptors for the canonical/B-catenin pathway in the breast. The following summarizes the work completed to date encompassing Objectives 1-3 of the original proposal.

1. Expression analysis of Frizzled and LRP expression at different stages of mammary development and in cells that respond or do not respond to Wnt4

The aim of this section was to determine which Frizzled proteins and which LRPs were expressed in RNA harvested from the mammary glands from virgin mice, from 12 day pregnant mice and from postpartum mice. Additionally, we aimed to extend and refine our original RT-PCR data set to include more cell lines that responded to Wnt4 or did not repond to Wnt4. This analysis has been completed for the varying stages of mammary development and for additional lines of responder and non-responder cells. The results indicate that several members of the Frizzled family are expressed at all stages of mammary development and in both responding and non-responding cell lines. The LRPs were ubiquitously expressed, making Frizzleds the more likely candidates for signaling specificity. However, since there is no individual Frizzled missing from all non-responders the expression analysis does not distinguish a leading candidate (Table 1).

2. Gene transfer of receptors into cell lines unresponsive to Wnt4

The aim of this section was to test the hypothesis that a missing Frizzled is the specific receptor required for activation of the canonical pathway by Wnt4. Since no one candidate was identified in the expression analysis, we transiently transfected each Frizzled candidate into 3T3 cells (non-responders). We assayed the consequences of this transfection by TOPFLASH assays of transcriptional activation. The TOPFLASH assay uses a synthetic TCF responsive promoter to drive luciferase expression. We found that transient transfection of either Frizzleds 5 or 9 enabled 3T3 cells to respond to Wnt4 (Figure 1). We then generated stable 3T3-derived lines that express the candidate receptors by infecting 3T3 cells with retroviruses that contain the full-length Frizzled cDNA sequences. These 3T3/Frizzled5 and 3T3/Frizzled9 cells were then tested for changes in levels of cytosolic B-catenin and changes in TOPFLASH expression after incubation with Wnt4 conditioned medium. We found that expression of Frizzled5 or Frizzled9 in 3T3 cells enabled Wnt4 to activate canonical Wnt signaling (Figure 2). These experiments

suggest that elevated expression of Frizzled5 or Frizzled9 is sufficient to mediate Wnt4/β-catenin signaling in these cells. We are currently using RNAi to determine if they are necessary for Wnt4/β-catenin signaling in 10T1/2 cells.

3. Biochemistry to determine specific binding of Wnt4 to candidate receptors

Plasmids encoding fusion proteins composed of the cysteine rich domain (CRD) of Frizzled 5 and the Fc portion of the IgG heavy chain have been constructed and we are currently constructing a similar fusion protein encoding the CRD of Frizzled 9. Using this approach we have already determined that Frizzled8 can bind to Wnt4 and we are investigating whether Frizzled5 also binds. This is a crucial step before making a dominant negative form of the receptor for expression in transgenic mice.

We have established in vitro conditions for ligand-receptor crosslinking by exploiting our knowledge of Wnt4 binding to FrizzledCRD-IgG. These conditions are currently being used to approach the identification of cell surface receptors for Wnt4 by using chemical crosslinking as a complementary approach.

We are also in the process of identifying regions of Frizzled 5 and Frizzled 9 that are important for binding and signaling. We have compared all Frizzled sequences and have made maps of homology regions. These regions have been helpful as we construct a series of Frizzled 5 deletion constructs and a membrane anchored version of Frizzled 5.

Key research Accomplishments

- A comparison of Frizzled and LRP expression during mammary gland development and in cell lines indicates that Frizzled are the components more likely involved in signaling specificity for Wnt4
- Gene transfer of all Frizzleds into cells that do not respond to Wnt4 by activating indicate that Frizzled5 or Frizzled9 is sufficient for Wnt4/B-catenin signaling.
- Biochemical approaches to understanding binding and signaling have been begun by making deletion constructs and by using an in vitro IgG fusion protein approach
- Considerable time has been spent learning how to create/design/choose constructs for expression in transgenic mice by working with Keith Brennan, PhD who has made a Extracellular version of Frizzled8 cloned downstream of the mouse mammary tumor virus (MMTV) promoter
- Much effort has been spent investigating the mammary phenotype of transgenic mice. This effort has been a great educational experience and will serve me well once

mammary specific dominant negative Wnt4 receptor transgenic mice have been generated.

Reportable Outcomes

Manuscripts in preparation
K.R. Brennan, J.M. Gonzalez-Sancho, L.A. Castelo-Soccio, L.R. Howe and A.M.C Brown Truncated mutants of the putative Wnt receptor LRP6/Arrow can stabilize β-catenin independently of Frizzled proteins

L.A. Castelo-Soccio, K.R. Brennan, J.M. Gonzalez-Sancho and A.M.C. Brown Frizzled Proteins affect the Specificity of Intracellular Signaling in Response to Wnt4

Abstracts
K. Brennan, J.M. Gonzalez-Sancho, L.A. Castelo-Soccio, L.R. Howe, and A.M.C. Brown LRP5&6 can signal independently of Frizzled. 'Signalling the Future, 1902-2002' University of Liverpool, U.K., 2002

L. A. Castelo-Soccio, K. R. Brennan, and A.M.C. Brown Expression of a dominant negative Wnt receptor inhibits ductal outgrowth and alveolar hyperplasia in mouse mammary glands 94th Annual AACR (American Association of Cancer Research) Meeting, Toronto, Canada, 2003

Awards:
2003 AACR Minority Scholar Award in Cancer Research

Conclusions

We have conducted an expression profile analysis of Frizzled and LRP proteins at different stages of mammary development and in different cell lines that respond to Wnt 4 by activating canonical signaling or showing no response to Wnt 4. Our studies indicate that Frizzled proteins are most likely critical for signaling specificity. We have used transient transfections of Frizzled cDNAs into non-responding cells to identify candidate Wnt4 receptors so that we may identify methods of antagonizing the harmful effects of excess Wnt signaling in the breast. By transient transfection, we identified two candidate receptors. By stable transfection of these candidates into non-responder cells we have confirmed that both Frizzleds 5 and 9 are sufficient for Wnt4/β-catenin signaling using assays of cytosolic β-catenin and TCF transcriptional activation. Biochemical assays of binding of Frizzled 5 and Frizzled 9 will confirm binding of Wnt 4 to these candidates and enable us to make a decision about which candidate will be best for planned dominant negative Wnt4 receptor transgenic mouse experiments.

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Cadigan, K.M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev* **11,** 3286-305.

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Nusse, R. and Varmus, H.E. (1992). Wnt genes. *Cell* **69**, 1073-87.

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Appendices

Figures 1-2 , Table 1 and associated legends

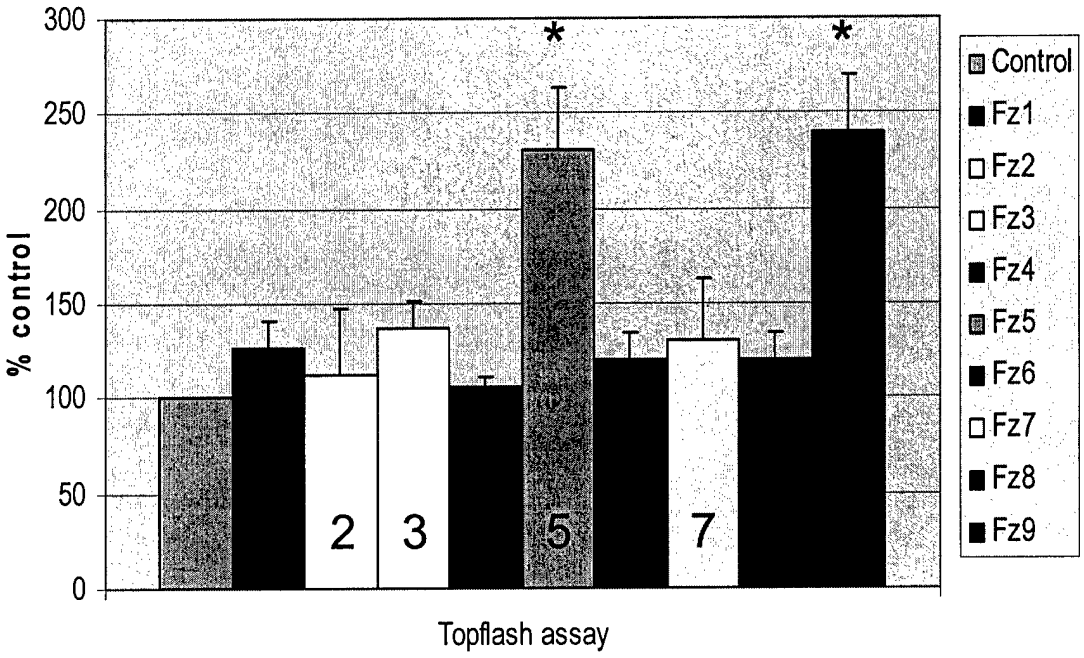
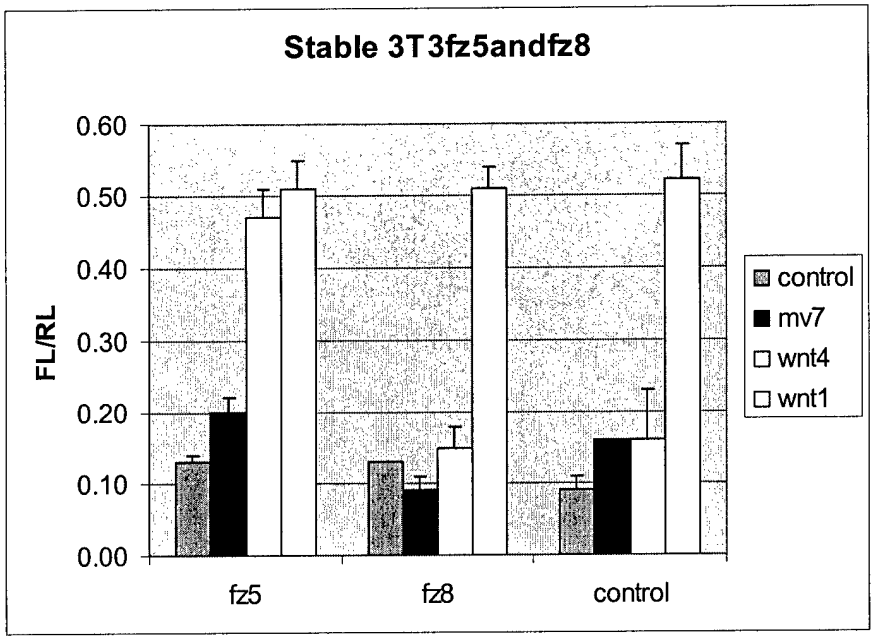


Figure 1: Overexpression Frizzled proteins in NIH3T3 cells
Topflash Luciferase assay. NIH3T3 cells stably expressing Wnt4 were co-transfected with one of 9 Frizzleds and the Topflash luciferase reporter and assayed after 36 hours for changes in luciferase activity. Co-tranfection of Frizzled 5 or Frizzled 9 leads to increased luciferase activity compared to controls. Bars represent the mean +/- SD of triplicate samples of a representative experiment (Students T-test p>.001).

A.



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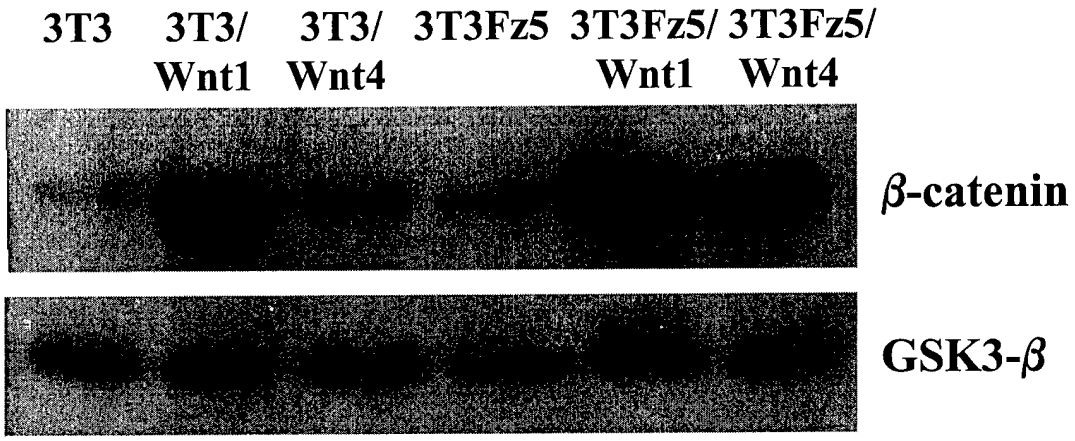

Figure 2. NIH 3T3 cells stably expressing Frizzled 5 or 9 are sufficient for Wnt4 to activate canonical signaling **A**.Top-Flash luciferase assay. NIH3T3 cells stably expressing either Frizzled 5 or Frizzled 8 were co-transfected with Wnt4 and the Topflash luciferase reporter. Cells expressing Frizzled 5 showed increases in luciferase activity in the presence of Wnt4. Control cells and cells expressing Frizzled 8 did not show an increase in luciferase activity. **B.** Western Blot analysis of cytosolic fraction of NIH3T3 cells stably expressing Frizzled 5 treated with Wnt1 or Wnt4 CM. GSK3-β serves as a loading control.

Table 1: Expression Profile of Frizzled proteins and LRP proteins in developing mammary gland (6 week virgin, 12 day pregnant and 12 day postpartum) and in four different cell lines. Positive and negative signs in parathenses next to cell lines indicate whether Wnt 4 activates canonical signaling in those cell lines. NIH3T3 and C57MG are non-responders. 10T1/2 and C2C12 cells are responsive to Wnt4.

Gene	Virgin	Pregnant	Postpartum	NIH3T3(-)	C57MG(-)	10T1/2(+)	C2C12(+)
LRP5	+	+	+	+	+	+	+
LRP6	+	+	+	+	+	+	+
Frizzled1	+	-	-	+	-	+	+
Frizzled2	+	+	+	+	+	+	+
Frizzled3	+	+	+	+	-	+	+
Frizzled4	+	+	+	+	+	+	+
Frizzled5	+	+	+	+	+	+	+
Frizzled6	+	+	-	-	+	+	+
Frizzled7	-	-	-	+	+	+	+
Frizzled8	+	-	-	-	+	+	-
Frizzled9	+	-	+	+	-	+	+